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Comparative functional analysis of the Rac GTPases

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Abstract **Small GTPases of the Rho family including Rac, Rho and Cdc42 regulate different cellular processes like reorganization of the actin cytoskeleton by acting as molecular switches. The three distinct mammalian Rac proteins share very high sequence identity but how their specificity is achieved is hitherto unknown. Here we show that Rac1 and Rac3 are very closely related concerning their biochemical properties, such as effector interaction, nucleotide binding and hydrolysis. In contrast, Rac2 displays a slower nucleotide association and is more efficiently activated by the Rac-GEF Tiam1. Modeling and normal mode analysis support the idea that altered dynamics of Rac2 at the switch I region may be responsible for different biochemical properties. These results provide insight into the individual functionalities of the Rac isoforms.**

Key words: Rac1; Rac2; Rac3; Rac isoform; T-lymphoma invasion and metastasis; GTPase

1. Introduction

The guanosine triphosphate (GTP) binding proteins of the Rho family (or Rho-GTPases) regulate a variety of cellular processes in all eukaryotic cells, ranging from cytoskeletal reorganization and cell motility to gene transcription in response to external stimuli [1]. As molecular switches, Rho-GTPases cycle between an inactive guanosine 5′-diphosphate (GDP)-bound state and an active GTP-bound state, and are strictly controlled by three classes of regulatory proteins [2]. While the guanine nucleotide exchange factors (GEFs) activate Rho-GTPases by stimulating the exchange reaction of bound GDP for GTP, GTPase activating proteins (GAPs) accelerate the slow intrinsic rate of GTP hydrolysis by some orders of magnitude, leading to their inactivation. Guanine nucleotide dissociation inhibitors (GDIs), on the other hand, bind to the GDP-bound form of Rho proteins and sequester them from the membrane to the cytoplasm.

The three distinct mammalian Rac isoforms (Rac1, 2 and 3), that are encoded by different genes, share between 89 and 93% identity in their respective amino acid sequence [3,4]. Rac1, the best-investigated isoform, regulates gene expression, cell cycle progression and rearrangement of the actin cytoskeleton [5]. Rac2 is proposed to be responsible for the regulation of the oxidative burst in hematopoietic cells but it has been shown that Rac1 contributes to this process, too [6]. Rac1 and Rac3 are ubiquitously expressed and therefore regulate a wide variety of cellular processes, whereas Rac2 is predominantly expressed in cells of the hematopoietic lineage. However it still remains unclear how the specificity of Rac isoforms is achieved in spite of the high degree of amino acid identity. In addition, it is not known, whether the mammalian Rac isoforms have different biochemical characteristics. In the present study we investigated their intrinsic activities such as nucleotide binding, GTP hydrolysis and their interaction with the Rac-specific GEF Tiam1 (T-lymphoma invasion and metastasis) and the downstream effector PAK (p21 activated kinase). It turned out that Rac2 has different biochemical characteristics compared to Rac1 and Rac3 concerning nucleotide association and Tiam1-accelerated nucleotide exchange.

2. Materials and methods

2.1. Plasmids

Human Rac1 (NM_006908), Rac2 (NM_02872) and Rac3 (AF008591), Cdc42 (NM_001791) and RhoA (aa 1–181; L25080) were cloned in pGEX4T1 via *Bam*HI/*Eco*RI sites and the DH-PH domain of Tiam1 (aa 1033–1404; U05245) via *Bam*HI and *Xho*I sites. pGEX-αPAK-GBD (aa 57–141; NM_002576) was kindly provided by J. Collard [7].

2.2. Proteins

Rac1, 2 and 3, Cdc42, RhoA, DH-PH domain of Tiam1, and the αPAK-GBD were produced as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* as described before [8]. GST-fusion proteins were purified by glutathione sepharose affinity chromatography with subsequent Thrombin cleavage, concentration with centrifugal concentrators (Vivaspin 10 kDa MWCO, Viva Science) and size exclusion chromatography (Superdex 75, Pharmacia, Uppsala, Sweden) to obtain a final purity of at least 95%. Nucleotide-free Rac proteins were prepared using enzymatic activity of alkaline phosphatase (Roche) and phosphodiesterase (Sigma) at 4°C [9]. GppNHp (guanosine 5′-β,γ-imidotriphosphate)-, mantGDP- (2′,3′-*O*-*N*-methylanthraniloyl-GDP) and mantGppNHp-bound Rac proteins were prepared by mixing nucleotide-free form of the respective proteins and the fluorescent nucleotides in a molar ratio of 1:1.5 and by using prepacked gel-filtration column (NAP5, Pharmacia) to remove unbound nucleotides. The concentrations of nucleotide-bound proteins were determined by high-performance liquid chromatography (HPLC) as described [8].

2.3. Nucleotide association and dissociation reactions

Association of mantGDP to nucleotide-free Rac proteins was car-

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Abbreviations: GBD, GTPase binding domain; GEF, guanine nucleotide exchange factor; GDP, guanosine 5′-diphosphate; GppNHp, guanosine 5′-β,γ-imidotriphosphate; mantGDP, 2′,3′-*O*-*N*-methylanthraniloyl-GDP; GppNHp, guanosine 5′-β,γ-imidotriphosphate; mantGppNHp, 2′,3′-*O*-*N*-methylanthraniloyl-GppNHp; PAK, p21 activated kinase; Tiam, T-lymphoma invasion and metastasis

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ried out at 20°C in 30 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 10 mM Na_2HPO_4/NaH_2PO_4, 3 mM DTE using a stopped flow apparatus (applied photophysics, SX16MV, Surrey, UK) as described for hGBP1 [8]. Intrinsic and Tiam1 DH-PH-stimulated dissociation of mantGDP from Rac proteins was monitored by spectrofluorometry (LS50B Perkin Elmer spectrofluorometer, Norwalk, CT, USA) and by stopped flow analysis as originally described for Cdc25 and Ras [10]. The observed rate constants were fitted using the Grafit program (Erithacus software).

2.4. GTPase assay

The GTP hydrolysis reaction was measured with a mixture of 80 μM nucleotide-free GTPase and 70 μM GTP at 25°C in 30 mM Tris–HCl pH 7.5, 5 mM DTE, 10 mM Na_2HPO_4/NaH_2PO_4, 10 mM $MgCl_2$ by HPLC as described before [8,11]. Exponential fitting of the data was done using Grafit (Erithacus software).

2.5. GDI measurements

The inhibition of mantGppNHp release from Rac proteins (0.2 μM) by increasing amounts of PAK-GBD in the presence of unlabeled GppNHp (40 μM) was monitored on LS50B Perkin Elmer spectrofluorometer (Norwalk, CT, USA) according to Herrmann et al. [12].

2.6. Molecular modeling and normal mode analysis (NMA)

The fluctuations of Cα atoms were calculated by NMA implemented as a VIBRAN module in the program CHARMm. The structure of Rac1 was taken from its crystal structure [13]. The Rac2 and Rac3 structures were created changing the differing amino acids in the Rac1 structure maintaining the rest of the structure unchanged. All structures containing nucleotide, magnesium and the crystallographic waters were minimized and subjected to the NMA.

3. Results and discussion

3.1. Biochemical properties of the three Rac isoforms

Small GTPases such as Rac1, Rac2 and Rac3 bind guanine nucleotides and hydrolyze GTP to GDP. For a better understanding how functional specificity of the three Rac isoforms is achieved, we first addressed nucleotide binding capabilities of the three Rac isoforms by fluorescence spectroscopic methods utilizing GTPase-bound fluorescently labeled mantGDP [8]. As shown in Fig. 1A, the mantGDP displacement from Rac proteins by excess amounts of non-labeled GDP was very slow ($0.7–1.2\times10^{-4}$ s^{-1}) and nearly similar for all three isoforms. Fig. 1B is a control experiment that shows that addition of excess EDTA, which depletes magnesium ions, leads to complete spontaneous nucleotide release. To gain insight into nucleotide binding affinity we measured the association kinetics of mantGDP to nucleotide-free Rac proteins. Direct comparison of mantGDP association to all three isoforms by stopped flow analysis revealed a drastically reduced association rate for Rac2 as shown in Fig. 1C. From the analysis of the observed rate constants presented in Fig. 1D, the association rate constant (2.2×10^6 M^{-1} s^{-1}) for mantGDP binding to Rac3 was determined. Unlike Rac1, which resembled Rac3 with respect to nucleotide association (unpublished data), we observed an about 13-fold slower rate constant for the mantGDP association to Rac2 (0.17×10^6 M^{-1} s^{-1}). Interestingly, the nucleotide association was not significantly

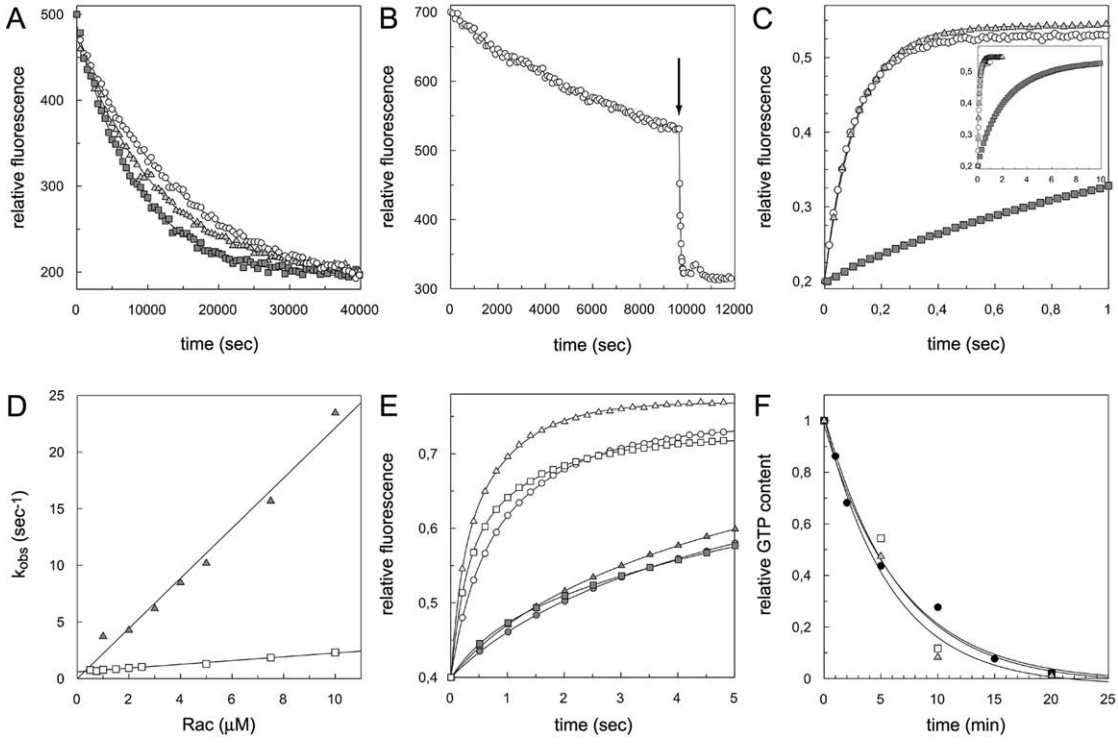

Fig. 1. Biochemical properties of the Rac proteins. A: Intrinsic mantGDP dissociation from 0.1 μM Rac1 (open circles), Rac2 (filled squares) and Rac3 (filled triangles) was monitored in the presence of excess amounts of non-labeled GDP. Collected data were single exponentially fitted to obtain the dissociation rates k_{off}. B: MantGDP dissociation from Rac1 (0.1 μM) in the presence of 40 μM GDP was completed after addition of 20 mM EDTA (arrow). C: MantGDP (0.1 μM) association to 5 μM nucleotide-free Rac1 (open circles), Rac2 (filled squares) and Rac3 (open triangles) was monitored. The inset shows the completed association reaction of mantGDP to nucleotide-free Rac2. D: The association rate constants (k_{on}) of mantGDP binding to Rac3 (filled triangles) and Rac2 (open squares) were obtained by linear fitting at increasing concentrations of the respective nucleotide-free proteins. E: Binding of 0.1 μM mantGDP to 1 μM Rac1 (open symbols) and Rac2 (filled symbols) was measured in the absence of Tiam1 and EDTA (circles), and in the presence of 0.5 μM Tiam1 DH-PH (squares) or 10 mM EDTA (triangles). F: GTP hydrolysis reaction of Rac1 (open circles), Rac2 (filled squares) and Rac3 (open triangles) measured by HPLC. The relative GTP content was single exponentially fitted to obtain the rate constant for the GTPase reaction of the respective Rac isoform.

influenced when the measurements were carried out in the presence of either the DH-PH domain of the RacGEF Tiam1 or magnesium-chelating EDTA (Fig. 1E). These data suggests that the presence of GEF or the absence of magnesium ions does not affect nucleotide association to Rac proteins.

Dissociation constants (K_d) were calculated from the kinetic parameters of dissociation and association reactions. Due to the fact that the nucleotide dissociation rate is nearly similar for all three isoforms ($0.7–1.2\times10^{-4}$ s^{-1}), we obtained a 17-fold higher nucleotide affinity for Rac3 ($K_d = 0.041$ nM) as compared to that of Rac2 ($K_d = 0.706$ nM). The different nucleotide binding properties of Rac1/Rac3 and Rac2 may be caused by different conformational flexibilities around their switch I regions (3.4). Nonetheless, the very high overall affinity of Rac proteins for mantGDP, determined in this study, is in the same range as reported before for the members of other small GTPase families such as Ras, Ran and Rab [9,14,15]. However, GDP affinity of Rac1 has been previously reported to be at a submicromolar concentration ($K_d = 0.62$ μM) [16] that is 20 000-fold lower than what was determined in this study. This discrepancy is due to the different method used for investigating the nucleotide association. Unlike the single time point (6 h) filter binding assay [16], our studies are based on individual time-resolved fluorescence measurements of the primary steps of association and dissociation and thereby are likely to be more precise.

Intrinsic GTP hydrolysis reaction of small GTPases is a second crucial function, which was examined for the respective Rac isoforms by measuring single turnover GTPase reaction using HPLC. As shown in Fig. 1F, the Rac proteins hydrolyzed GTP with a similar intrinsic rate of 0.14 min^{-1}, which resembles the GTPase reactions of RhoA and Cdc42 (data not shown) but is about five-fold faster than that of Ras proteins [11]. Comparison of the corresponding structures did not provide any hint why Rho GTPases display a higher GTP hydrolysis rate than Ras proteins. The Rac isoforms contain an identical GAP binding interface [17], suggesting that the basic mechanism of the GAP-stimulated GTPase reaction is most likely conserved [18].

3.2. Rac2 as the most specific Tiam1 substrate

Although Tiam1 has been the subject of extensive investigations, the Tiam1-stimulated nucleotide exchange reaction of Rac proteins has not been quantitatively analyzed so far. In this study we investigated the nucleotide exchange activity of Tiam1 DH-PH (aa 1033–1404) on different GTPases of the Rho family, using a fluorescence spectroscopic assay [8]. As shown in Fig. 2A, the very slow intrinsic dissociation of bound mantGDP from Rac1 is accelerated about 11-fold (0.00077 s^{-1}) in the presence of the DH-PH domain of Tiam1. However, this was not observed for the closely related GTPases RhoA and Cdc42 (Fig. 2B,C), confirming the previous data from filter binding assay that Tiam1 is a Rac-specific exchange factor [19]. Recently, it has been shown that W56 of Rac1 (F56 in Cdc42) is a necessary and sufficient binding determinant of Rac1 for the discrimination by a subset of Rac-specific GEFs such as TrioN, GEF H1 and Tiam1 [20,21]. Interestingly, mutations of W56F in Rac1 and F56W in Cdc42 inverted the Tiam1 specificity from Rac1 to Cdc42 and the specificity of the Cdc42 GEF intersectin from Cdc42 to Rac1 [20,21]. As W56 is present in all three Rac isoforms, Tiam1 is likely to recognize all three GTPases as substrates. We therefore measured the activity of the Tiam1 DH-PH domain on the isoforms Rac2 and Rac3 under the same conditions as for Rac1. Whereas Tiam1-induced nucleotide exchange of Rac3 (0.00075 s^{-1}) was equal to that of Rac1, the kinetic of the mantGDP dissociation reaction from Rac2

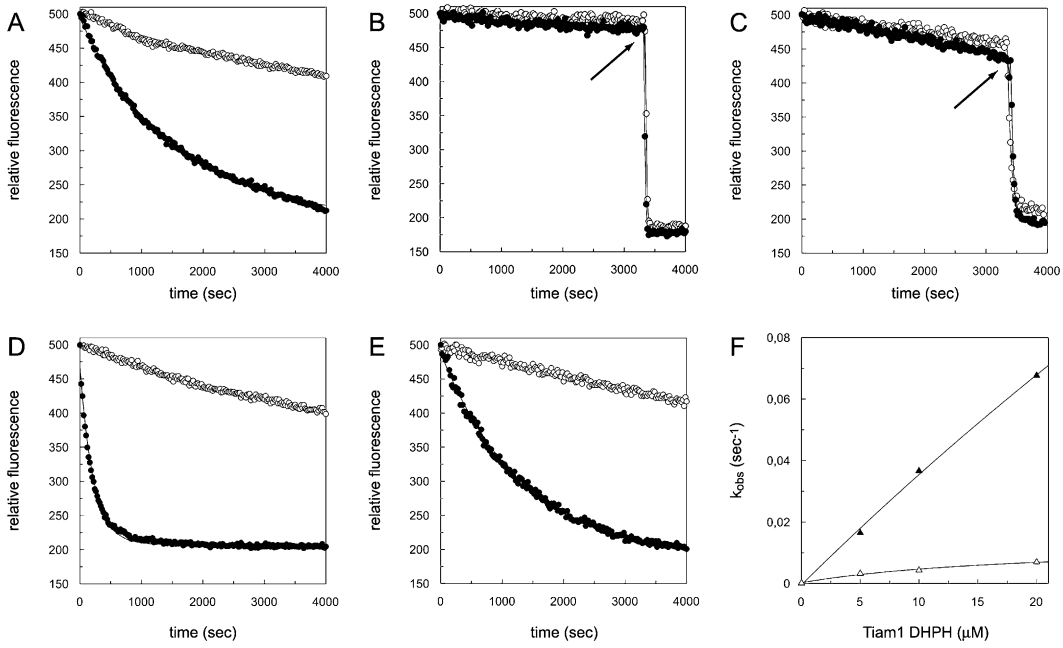

Fig. 2. Stimulation of nucleotide dissociation by the DH-PH domain of Tiam1. MantGDP displacement from 0.1 μM Rac1 (A), RhoA (B), Cdc42 (C), Rac2 (D) and Rac3 (E) was monitored after the addition of 20 μM unlabeled GDP in the absence (open circles) and in the presence of 0.5μM Tiam1 DH-PH (filled circles). Arrows indicate EDTA addition (40 mM) at a given time point as a control for accelerated nucleotide release. F: For Rac1 and Rac2 the dependence of the observed rate constants on Tiam1 DH-PH concentration was determined. By single exponential fitting of the resulting curves, kinetic parameters were determined.

($0.0045\ s^{-1}$) was about six-fold faster (Fig. 2D,E). The Tiam1-stimulated exchange reaction was next quantified for Rac1 and Rac2. Extrapolation of observed rate constants with increasing DH-PH concentrations resulted in an up to 5900-fold increase in the nucleotide dissociation rate of Rac2 and 190-fold for Rac1, as compared to the intrinsic rates of either GTPase. The observed rate constants were plotted against the various DH-PH concentrations to estimate the kinetic parameters of the Tiam1-stimulated nucleotide dissociation from Rac1 ($K_d = 20$ μM, $k_{max} = 0.013\ s^{-1}$) and Rac2 ($K_d = 187$ μM, $k_{max} = 0.706\ s^{-1}$) (Fig. 2F). The Tiam1 exchange reaction in the comparison to other Rac-specific GEFs [20] is rather slow on Rac1 and Rac3 but attains approximately similar rates on Rac2, suggesting Rac2 as the most specific Tiam1 substrate in vitro. As Tiam1 does not significantly influence the association reaction (Fig. 1E), the higher Tiam1 activity on Rac2 is not due to a slower nucleotide association.

3.3. PAK binds similarly to the Rac isoforms

To quantitatively analyze the specificity of the three Rac isoforms for PAK-GBD as a representative GTPase effector, the interaction of the PAK-GBD protein (PAK_{57-141}) with respective GppNHp (non-hydrolyzable GTP analog)-bound Rac proteins was measured using a fluorescence-based GDI assay. This assay, which has been established for studies of Ras-effector interactions [22], uses the ability of effector proteins to inhibit the nucleotide dissociation from the GTPase. Accordingly, increasing amounts of PAK_{57-141} resulted in an incremental inhibition of the mantGppNHp release from Rac1, even in the presence of excess amounts of non-labeled GppNHp. The observed rate constants were plotted against the applied PAK_{57-141} concentrations and the data were fitted to obtain a K_d of 610 nM for the Rac3-PAK_{57-141} interaction (Fig. 3A). Employing the same assay as for Rac3 we observed comparable rate constants of the inhibition of mantGppNHp dissociation from Rac2 (Fig. 3B) and Rac1 (Fiegen et al., J. Biol. Chem., in press) at increasing PAK concentrations. By fitting of this data we obtained a K_d of 130 nM for the Rac2-PAK_{57-141} interaction (Fig. 3B). This data indicates that the Rac isoforms employ a similar mode of interaction with PAK – most likely by forming a β-strand connection to the switch I region as shown for the Cdc42-PAK interaction [23].

3.4. Increased conformational flexibility of the switch I and the insert helix

Although Rac proteins share 92% sequence identity, our data indicate that the isolated DH-PH domain of Tiam1 is more effective on Rac2 than on Rac1 or Rac3 in vitro. There are six amino acid deviations in the G domain of Rac2 compared to Rac1 and Rac3, which are located neither in the vicinity of switching regions (Fig. 4A), neither at the nucleotide binding site nor at the contact site between Rac and Tiam1 [24]. Varying properties observed for Rac2 can therefore not be explained by the direct influence of amino acid substitutions. These differences can rather be explained by changes in the flexibility of protein parts that spread from few altered amino acids all over the molecule. The fluctuations of Cα atoms for Rac1, Rac2 and Rac3 using the NMA module of the CHARMm program were calculated and we found differences within the switch I region, the insert region and the very C-terminal end (Fig. 4A), although they contain identical

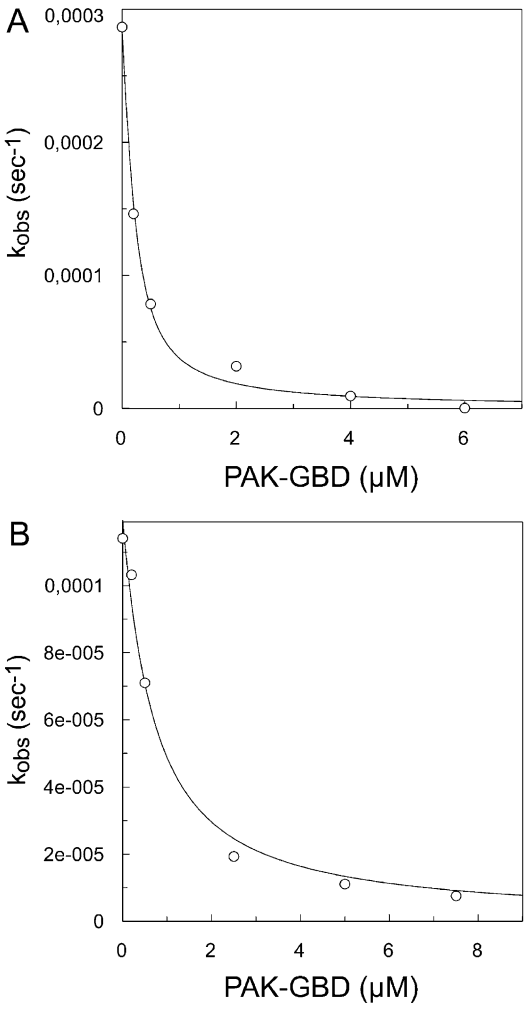

Fig. 3. Quantitation of Rac binding to PAK-GBD. Observed rate constants obtained by PAK-GBD binding to Rac3 (A) and Rac2 (B) in the GDI assay were fitted to obtain K_d values for the effector interaction.

amino acids. Assuming that the correlated motion between the ligand and the active site of the protein facilitates their interaction, the higher flexibility of the switch I regions of Rac1 and Rac3 in comparison to Rac2 may explain the functional differences of these proteins. On the other hand, it was shown [24] that the interactions of β2/β3 and switch II of Rac1 with Tiam1 enable the alteration of the switch I conformation facilitating subsequent nucleotide release. The relative rigidity of switch I in Rac2 may be a decisive factor for higher Tiam1 activity on Rac2 as described above.

The observed higher mobility of the helical insert region between β5 and α4 (Fig. 4A) was also found in the X-ray structure of Rac1 [13]. Interestingly, it has been shown that the insert region is essential for the catalytic activity of the Rac effector NADPH oxidase [25].

The variable highly mobile C-terminal end, which harbors the polybasic region and the posttranslational modification site (CAAX motif), was found to be disordered in Rac1 [13] and many structures of small GTPases. It can be assumed that the flexible tail is fixed upon its anchoring into the particular membrane compartment that is required for biological functions of these proteins.

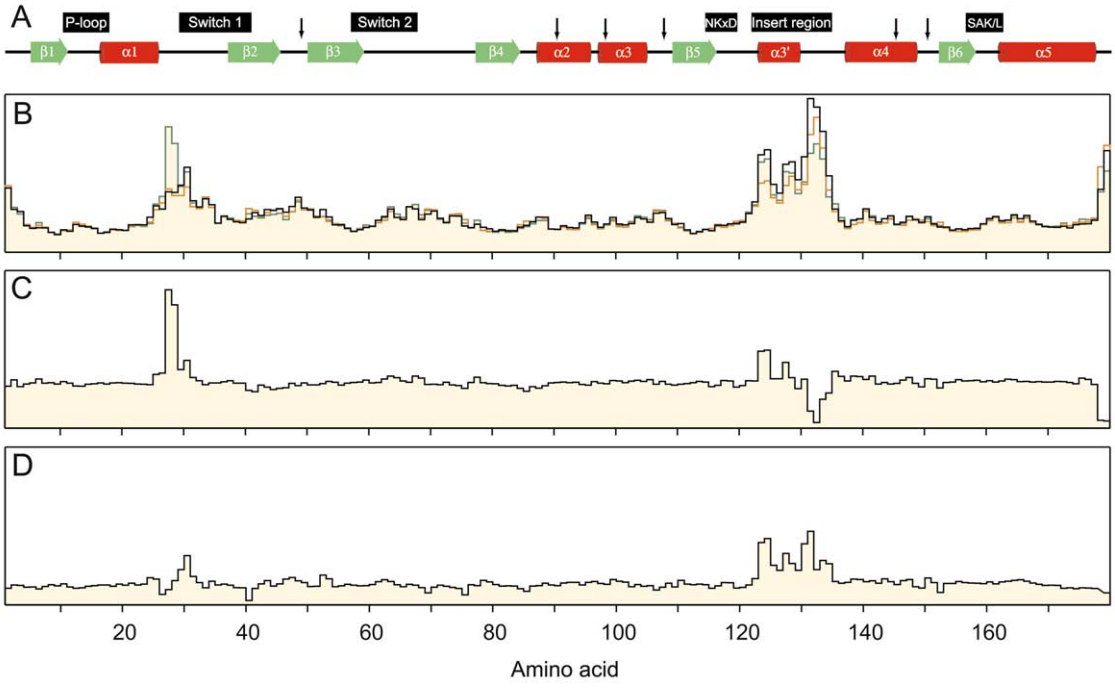

Fig. 4. Dynamics of Rac isoforms. A: Secondary structure elements and conserved sequence motifs of the Rac proteins. Secondary structures are illustrated as red barrels (α-helices) and green arrows (β-strands), respectively. Black boxes show characteristic motifs conserved in Rac proteins. Amino acid deviations in Rac2 are indicated by arrows. B: NMA of Rac1 (green), Rac2 (red) and Rac3 (black) structural models revealed different conformational flexibilities that are clustered in proximity to the switch I region and the insert helix. C,D: Differential plots. The normal modes of Rac2 shown in B were subtracted from Rac1 (C) or Rac3 (D), to show differences in mobility. Maxima indicate a higher Rac1/3 fluctuation and minima indicate a higher Rac2 mobility. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Concluding remarks

In the present study we addressed the specificity of nucleotide binding and effector interaction of the highly related Rac isoforms. We observed that the three highly related GTPase homologues, Rac1, Rac2 and Rac3 exhibit consistent biochemical characteristics such as GTP hydrolysis and effector binding, but also different properties regarding nucleotide binding and their activation by Tiam1. Thus, from our data, we conclude that the dynamics of these proteins are likely to account for their functional specificity.

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